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Suppression of myocardial ischemia–reperfusion injury by inhibitors of cytochrome *P450* in rats

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ABSTRACT

The effects of inhibitors of cytochrome *P450* on myocardial regional ischemia–reperfusion injury were examined in rats. Ischemia–reperfusion injury was evoked by ligation of the left anterior descending coronary artery for 1 h, followed by reperfusion for 24 h. Injuries were evident in causing infarction, decreases in left ventricular systolic pressure and left ventricle (dP/dt max)/P and an increase in left ventricular end-diastolic pressure. Increases in lipid peroxidation and reactive oxygen species levels in the ischemic region were observed. Intravenous injection of the potent cytochrome *P450* inhibitor sulfaphenazole at 10 and 30 mg/kg at the time of reperfusion reduced infarct size by 41.7 and 73.2%, respectively; and improved cardiac function accompanied by the decrease in content of lipid peroxide and reactive oxygen species in the area at risk. Cardiac testosterone metabolism was inhibited by sulfaphenazole administration, indicating its inhibitory effects on cardiac cytochrome *P450* activity. Another cytochrome *P450* inhibitor, cimetidine, given intravenously, had similar effects to sulfaphenazole on reperfusion injury. Taken together, these results indicate that reactive oxygen species derived from cytochrome *P450* play an important part in myocardial regional ischemia–reperfusion injury *in vivo*, and strongly support the hypothesis that cytochrome *P450* inhibitors are promising therapeutic agents for cardiac ischemia–reperfusion injury.

1. Introduction

Various studies have shown that the production of reactive oxygen species in cardiomyocytes undergoing ischemia and/or reperfusion is one of the major events that ultimately results in myocardial infarction (Kloner et al., 1989). The multitude of reactive oxygen species generated during the oxidative stress associated with ischemia and/or reperfusion can damage cellular components such as membrane lipid, protein and DNA, leading to myocardial cell death. NADPH oxidase (Chen et al., 2007; Tao et al., 2007), xanthine oxidase (Thompson-Gorman and Zweier, 1990), cyclooxygenase (Janero et al., 1989), lipoxygenase (Janero et al., 1989), inducible nitric oxide synthase (Zhao et al., 2007) and mitochondrial respiratory chain (Levraut et al., 2003) are reported to be potential reactive oxygen species sources during ischemia and/or reperfusion.

Cytochrome *P450* family proteins are monooxygenases, which are primarily located in the endoplasmic reticulum in hepatocytes. They catalyze the oxidation of many exogenous and endogenous compounds (Gonzalez, 1988). Attempts to explore the functions of cytochrome *P450*s (other than the role for metabolism) have progressed. This includes reactive oxygen species production, which has been highlighted in recent years. Cytochrome *P450*s produce reactive oxygen species *via* their catalytic cycle with or without substrate metabolism (Bondy and Naderi, 1994; Rashba-Step and Cederbaum, 1994; Zangar et al., 2004). It has recently been reported that reactive oxygen species generated from cytochrome *P450*s are an important factor in the pathogenesis of Parkinson's disease (Shahabi et al., 2008), diabetes (Raza et al., 2004) and hepatic fibrosis (Nieto et al., 2002).

Contents of cytochrome *P450*s in the heart are ≤10% of those in the liver (Guengerich and Mason, 1979). The predominant role of cardiac cytochrome *P450*s is to metabolize arachidonic acid (Roman, 2002). Cardiac cytochrome *P450*s metabolize arachidonic acid to biologically active eicosanoids, epoxyeicosatrienoic acids or hydroeicosatetraenoic acids by distinct enzymatic reactions. Granville et al. (2004), using a rat Langendorff preparation, recently showed that the cytochrome *P450* inhibitor sulfaphenazole reduced not only reactive oxygen species levels, but also myocardial injury during reperfusion after global ischemia, indicating that cytochrome *P450* is one of the sources of reactive oxygen species during reperfusion injury and contributes to the extension of infarct size. In experiments using the Langendorff model, blood factors contributing to reperfusion injury were not considered. Potential sources of reactive oxygen species after reperfusion of the coronary artery *in vivo* are mainly macrophage and neutrophils infiltrated from blood, which are known to be involved in ischemia–reperfusion injury in various tissues (in addition

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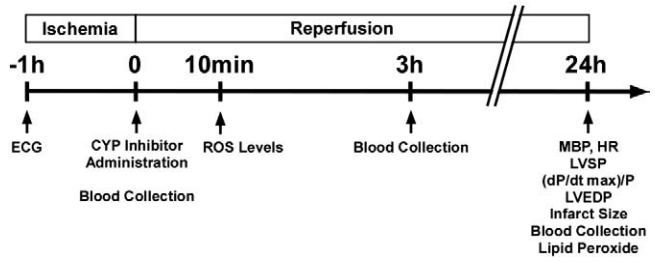

Fig. 1. Experimental protocol. The experimental protocol shows the time of acquisition of biochemical and physiological data, together with drugs administration. The time "0" denotes the time of reperfusion after 1-h occlusion of left anterior descending coronary artery and reperfusion ensues after 24 h. ECG: electrocardiograph, CYP: cytochrome *P450*, ROS: reactive oxygen species, MBP: mean blood pressure, HR: heart rate, LVSP: left ventricular systolic pressure.

to the above-mentioned sources) (Vinten-Johansen, 2004). These cells in blood invade ischemic tissues and are thought to induce inflammation and cardiomyocyte injury by reactive oxygen species production. The regional ischemia–reperfusion model *in vivo* is therefore considered to be closer to clinical settings than other models as reported by Granville's and Khan's groups (Granville et al., 2004; Khan et al., 2007).

We attempted to clarify the contribution of reactive oxygen species derived from cytochrome *P450*s to cardiac injury *in vivo* after reperfusion of the left anterior descending coronary artery in the rat. Furthermore, we investigated if cytochrome *P450* inhibitors could be therapeutic agents for cardiac reperfusion injury.

2. Materials and methods

2.1. Materials

Sulfaphenazole and 4-androstene-3,17-dione were obtained from Sigma-Aldrich (St. Louis, MO, USA). Cimetidine and testosterone were purchased from Wako Pure Industries Limited (Osaka, Japan). Dihydroethidium was purchased from Molecular Probes (Eugene, OR). All other chemicals were obtained from Sigma-Aldrich or Wako, and were of reagent grade.

2.2. Animals

Procedures were in accordance with the Animal Care and Use Committee of Tokushima Bunri University, Kagawa, Japan.

Male Wistar rats were purchased from Nippon CLEA (Osaka, Japan). Rats were bred at the animal facility of Tokushima Bunri University at Kagawa Campus, in a temperature-controlled room (25 °C) with a reverse 12:12-h light–dark cycle. After acclimation for 7–10 days at the animal facility, rats were used for experimentation.

2.3. Myocardial ischemia and reperfusion

Animal models of myocardial ischemia and reperfusion were prepared according to the method previously described with slight modifications (Watanabe et al., 1991). Briefly, rats were anesthetized with pentobarbital sodium (40 mg/kg, i.p.) and intubated. Under artificial ventilation with a ventilator (SAR-830/P, CWE Incorporated, Ardmore, PA, USA), left thoracotomy was done at the fourth intercostal space and the heart was exposed. The left anterior descending coronary artery was threaded 3–4 mm from its origin to make a snare. Myocardial ischemia was produced by occlusion of the left anterior descending coronary artery by tightening the snare. Rats underwent 1 h of ischemia. The myocardium was reperfused by releasing the snare. The thread was left untied and the chest was closed, and rats were allowed to recover from anesthesia and kept in the animal facility. Sham-operated rats were subjected to the same surgical procedures, except that the suture under left anterior descending coronary artery was not tied.

2.4. Drugs

Sulfaphenazole was dissolved in 0.1 M sodium hydroxide at 20 mg/ml, and cimetidine was dissolved in 0.5 M hydrogen chloride at 100 mg/ml. Both drugs were injected into the femoral vein immediately after the solutions were prepared.

2.5. Experimental protocol (described schematically in Fig. 1)

Left anterior descending coronary artery was occluded for 1 h and then reperfused for 24 h. The time "0" was defined as the starting point of reperfusion. Saline as a control vehicle, sulfaphenazole and cimetidine were administered into the femoral vein at 0 h. Blood was collected at 0, 3 and 24 h. Levels of reactive oxygen species were evaluated 10 min after reperfusion. Hemodynamic parameters were measured and infarct size and lipid peroxidation were evaluated 24 h after reperfusion. The measurements of infarct size and lipid peroxidation were done in different studies (Fig. 1).

2.6. Measurement of cardiovascular hemodynamics

The lead II electrocardiogram was continuously monitored *via* electrodes connected to an electrocardiograph signal transducer (AD Instruments Incorporated, CO, USA). Blood pressure was measured with a fluid-filled catheter connected to a pressure transducer (DH-100; Nihon Kohden, Tokyo, Japan) and inserted into the right carotid artery. Left ventricular pressure was monitored using a high-fidelity catheter-tip micro-manometer (2F; Millar Instruments, Incorporated, TX, USA), inserted into the right carotid artery, and advanced to the left ventricle. Electrocardiograph, mean blood pressure, left ventricular systolic pressure, heart rate, left ventricle (dP/dt max)/P and left ventricular end-diastolic pressure were recorded with the sampling frequency of 1 kHz on an eight-channel, multipurpose data-acquisition system (Power Lab; AD Instruments Incorporated) connected to a computer.

2.7. Evaluation of infarct size

After measurement of cardiovascular hemodynamics, the left anterior descending coronary artery was re-occluded at the same position used for induction of ischemia, and the heart excised. Evans blue (1% solution in saline) was injected into the aorta to visualize non-ischemic regions. Atria and the right ventricle were removed. The left ventricle was sectioned perpendicularly to the apex-base into slices (2-mm thickness) and incubated in 1% triphenyltetrazolium

Table 1
Weights of body and heart of rats.

	Sham	I/R	+10 mg/kg SPZ	+30 mg/kg SPZ	+10 mg/kg Cime	+30 mg/kg Cime
Body weight (g)	295 ± 10	286 ± 7	294 ± 9	301 ± 5	284 ± 6	298 ± 5
Heart weight (g)	0.88 ± 0.04	0.95 ± 0.05	0.99 ± 0.04	0.89 ± 0.05	0.88 ± 0.01	0.86 ± 0.01

Body weight was measured before surgery and 24 h after reperfusion. Heart weight was measured 24 h after reperfusion. Values are mean ± S.E.M. of seven separate experiments. Sham: sham-operated group, I/R: ischemia and reperfusion group, SPZ: sulfaphenazole, Cime: cimetidine.

Table 2
Weights of left ventricle and ischemic region.

	Sham	I/R	+10 mg/kg SPZ	+30 mg/kg SPZ	+10 mg/kg Cime	+30 mg/kg Cime
left ventricle weight (g)	0.63 ± 0.04	0.70 ± 0.04	0.70 ± 0.03	0.66 ± 0.03	0.61 ± 0.02	0.62 ± 0.01
Ischemic region (g)	0.30 ± 0.03	0.32 ± 0.04	0.32 ± 0.04	0.28 ± 0.04	0.26 ± 0.02	0.26 ± 0.02
Area at risk (%)	46.1 ± 3.1	45.0 ± 4.2	44.7 ± 5.4	40.8 ± 4.2	43.1 ± 2.8	41.3 ± 2.6

Weights of left ventricle and ischemic region were measured 24 h after reperfusion, and the area at risk calculated. Values are mean ± S.E.M. of seven separate experiments. Sham: sham-operated group, I/R: ischemia and reperfusion group, SPZ: sulfaphenazole, Cime: cimetidine.

chloride for 10 min at 37 °C. Triphenyltetrazolium chloride-stained slices were divided into three portions; viable tissue in the area at risk (stained red), infarct area (not stained) and non-ischemic area (stained blue). Each portion was weighed to determine infarct size. The weight of area at risk was equal to the weight of tissue stained red plus the weight of non-stained tissue. Infarct size was expressed as percentage of area at risk calculated from the following equation:

(weight of infarct area) / (weight of area at risk) × 100

2.8. Assay for lipid peroxidation

The left ventricle was frozen with a Wollenberger clamp pre-cooled in liquid nitrogen, and was powdered using Cryo Press (Microtec Company Limited, Chiba, Japan) at liquid nitrogen temperature. Powder was kept at −80 °C until measurement of lipid peroxide.

The content of thiobarbituric-acid-reactive substance was estimated using the method described by Ohkawa et al. (1979) with a slight modification and was used as an index of lipid peroxidation. Briefly, suspension was prepared by adding KCl solution to the powdered tissue followed by homogenization. The homogenate was mixed with sodium dodecyl sulphate, thiobarbituric acid, butylhydroxytoluene and acetic acid buffer. Mixture was incubated at 100 °C for 60 min, followed by rapid cooling and extraction with 1-butanol-pyridine. The absorbance of this extract at 532 nm was measured; 1,1,3,3-Tetraethoxypropane was the standard.

2.9. Plasma samples

At 0, 3 and 24 h during reperfusion, approximately 100 µl of whole blood was collected from the tail vein into a heparinized tube. It was centrifuged at 1500 ×*g* for 5 min to obtain plasma samples.

2.10. Determination of the concentration of cardiac troponin I in plasma

The concentration of cardiac troponin I in plasma was measured using High Sensitivity Rat Cardiac Troponin I ELISA Kit (Life Diagnostics Incorporated, PA, USA) according to the manufacturer's instructions. Briefly, cardiac troponin I in diluted plasma was sandwiched between solid-phase and horse radish peroxidase-conjugated antibodies. The absorbance at 450 nm was measured using tetramethylbenzidine as a substrate for horse radish peroxidase. The known concentrations of cardiac troponin I were the standard. All measurements were run in duplicates.

2.11. Determination of reactive oxygen species production

The levels of superoxide anion generated during reperfusion were estimated using the fluorescent dye, dihydroethidium (Laurindo et al., 2008). After reperfusion, 1 ml of dihydroethidium solution (2 mM in 20% DMSO) was administrated into left ventricle from apex. The heart was excised and non-ischemic regions were visualized by Evans blue injection. Tissues were cut into small pieces (~100 mm^3) placed in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan) and frozen in liquid nitrogen. Cryostat sections (20 µm) were cut out and mounted on glass slides. Confocal images were obtained with a Fluoview FV1000 confocal fluorescence microscope (Olympus, Tokyo, Japan) at 488 nm excitation, with a 560–660 nm band-pass filter.

2.12. Preparation of rat heart microsomal fraction

Rat heart microsomes were prepared by the method previously reported with slight modifications (Ishihara et al., 2006). Briefly, the rat heart was excised and cleared of blood and cut into small peaces, followed by homogenization in the buffer (50 mM Tris–HCl, pH7.4, 154 mM KCl, 1 mM EDTA, 1 mM DTT containing of protease inhibitor cocktail (Nakarai, Kyoto, Japan)) and centrifuged at 10,000 ×*g* for 20 min at 4 °C. The supernatant was re-centrifuged at 100,000 ×*g* for 60 min at 4 °C. The resulting microsomal pellet was suspended in 50 mM Tris–HCl buffer (pH 7.4) containing 20% glycerol, 1 mM EDTA, 1 mM DTT and protease inhibitor cocktail, and stored at −80 °C prior to use.

2.13. Measurement of testosterone metabolism

Testosterone metabolism was examined by the procedures reported previously with slight modifications (Mitsuda et al., 2006; Thum and Borlak, 2002). The reaction mixture consisted of 0.1 mg microsomal protein, 50 mM HEPES buffer (pH 7.4), 1.5 mM $MgCl_2$, 0.1 mM EDTA and 1 mM testosterone. After pre-incubation at 37 °C for 5 min, the reaction was started by the addition of NADPH at a concentration of 1 mM, and continued incubation for 3 h with gentle stirring at 37 °C. The reaction was terminated by the addition of ethyl acetate. Ethyl acetate layer was fractionated and dried under nitrogen gas. The dried residue was reconstituted in 25% (v/v) aqueous

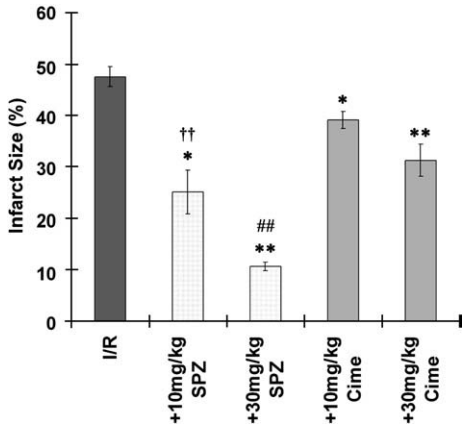

Fig. 2. Reduction of size of myocardial infarct by administration of cytochrome *P450* inhibitors. Left anterior descending coronary artery was occluded for 1 h and reperfused for 24 h. The heart was excised, followed by visualization of the area at risk by injection of Evans blue dye. Viable tissue and infarct size were evaluated by triphenyltetrazolium chloride staining. Values are given as mean ± S.E.M. of seven separate experiments. Data were analyzed using two-way analysis of variance, followed by the Student's *t* test with Holm's correction for multiple comparisons. $^{*}P<0.05$ and $^{**}P<0.01$ vs. I/R group. $^{\dagger\dagger}P<0.01$ vs. 10 mg/kg Cime-treated group. $^{\#\#}P<0.01$ vs. 30 mg/kg Cime-treated group. I/R: ischemia and reperfusion group. SPZ: sulfaphenazole, Cime: cimetidine.

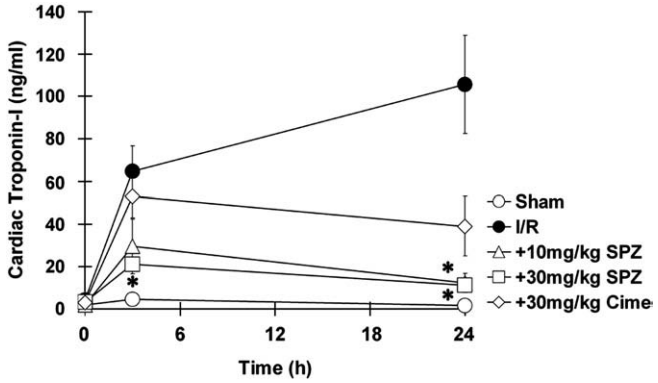

Fig. 3. Decreases in cardiac troponin I contents in plasma by administration of cytochrome *P450* inhibitors. Left anterior descending coronary artery was occluded for 1 h and reperfused for 24 h. Blood was collected from the tail vein at 0, 3 and 24 h, and cardiac troponin I contents in plasma measured. Values are mean ± S.E.M. of five separate experiments. Data were analyzed using Steel test with the Bonferroni correction for multiple comparisons. *$P<0.05$ vs. I/R group. Sham: sham-operated group. I/R: ischemia and reperfusion group. SPZ: sulfaphenazole, Cime: cimetidine.

methanol and assayed by HPLC. The known concentration of androstenedione was used as a standard.

The HPLC conditions were as follows: column, ODS-3 (3.0 × 250 mm, 4 μm) (GL Science, Tokyo, Japan); column temperature, 40 °C; flow rate 0.5 ml/min; UV detection, 246 nm. The mobile phase consisted of water (solvent A), methanol (solvent B) and acetonitrile (solvent C), and analysis was initiated with an isocratic elution of 60% A, 25% B and 15% C for 17 min, followed by 45% A, 40% B and 15% C for 6 min and finally 45% A, 45% B and 10% C thereafter. The total run time is 50 min per sample.

2.14. Determination of protein content

The protein content was determined according to the BCA method (BCA Protein Assay Reagent Kit, Pierce, IL, USA) using bovine serum albumin as a standard.

2.15. Statistical analyses

All data are expressed as means ± S.E.M. The effects of sulfaphenazole and cimetidine on the reduction of infarct size were analyzed using two-way analysis of variance, followed by the Student's *t*-test with Holm's corrections for multiple comparisons. Data obtained from three or more groups were compared using Dunnett's test or Steel test with the Bonferroni correction for multiple comparisons. $P<0.05$ was considered statistically significant.

3. Results

3.1. Weight of the body and heart

Body weight 24 h after reperfusion following 1-h occlusion of the left anterior descending coronary artery was not different between the groups, but a tendency toward a decrease in body weight in the ischemia–reperfusion group without drug treatment (untreated group) was noted (Table 1). Differences in heart weights between all groups were not observed (Table 1).

3.2. Reduction of myocardial ischemia–reperfusion injury by administration of cytochrome P450 inhibitors

Achievement of myocardial ischemia by left anterior descending coronary artery occlusion was ascertained by ST-segment elevation on the electrocardiograph (data not shown). Differences in the area at risk between all groups were not observed (Table 2).

Myocardial infarction was not observed in sham-operated rats (data not shown), indicating that surgical procedures did not cause myocardial infarction. Reperfusion for 24 h after ischemia for 1 h caused infarction in 47.5 ± 1.9% of the area at risk of the left ventricle (Fig. 2). Intravenous injection of sulfaphenazole, a cytochrome *P450* inhibitor, at 10 and 30 mg/kg at the time of reperfusion significantly reduced infarct size to 25.0 ± 4.2% and 10.6 ± 0.8%, respectively, in a dose-dependent manner (Fig. 2). Administration of cimetidine (a potent cytochrome *P450* inhibitor but structurally different from sulfaphenazole) at 10 and 30 mg/kg, also dose-dependently suppressed infarct size to 39.1 ± 1.6% and 31.2 ± 3.1%, respectively (Fig. 2). However, suppressive effects of sulfaphenazole at 30 mg/kg were more potent than those of cimetidine at 30 mg/kg and similar effects were observed at 10 mg/kg of both inhibitors. Thus, sulfaphenazole was more effective than cimetidine in suppression of reperfusion injury.

Because cardiac troponin I is a cardiomyocyte-specific soluble protein, release of this protein into blood is considered to reflect myocardial damage (Apple, 1992; O'Brien, 2008). The concentration of cardiac troponin I at 0, 3 and 24 h in plasma was about 2.0 ng/ml in normal and sham-operated rats, and there was no difference between them. In sham-operated rats, cardiac troponin I concentration in plasma showed no change 24 h after surgery (Fig. 3). Cardiac troponin I concentration in untreated rats was greatly increased 3 h after reperfusion and gradually increased thereafter, indicating continuous release of cardiac troponin I from damaged cardiomyocytes into blood (Fig. 3). The concentration of cardiac troponin I 24 h after reperfusion was 105.8 ± 23.2 ng/ml; a 25-fold increase compared with that at the time of reperfusion.

Sulfaphenazole administration dose-dependently inhibited the release of cardiac troponin I throughout 24 h of reperfusion (Fig. 3). Inhibition at 30 mg/kg was significant 3 h and 24 h after reperfusion, as shown by the decrease from 64.6 ± 12.0 to 21.1 ± 1.7 ng/ml, and from 105.8 ± 23.2 to 11.3 ± 2.8 ng/ml, respectively. Administration of cimetidine also suppressed cardiac troponin I release, although suppression was not significant because of the large variations of values measured.

3.3. Improvement of cardiac function by administration of cytochrome P450 inhibitors

Cardiovascular function was evaluated 24 h after reperfusion. Mean blood pressure in rats with ischemia and reperfusion was lower than that in sham-operated rats (Table 3). Mean blood pressure had a tendency to rise by administration of sulfaphenazole or cimetidine, whereas heart rate was not affected.

Left ventricular systolic pressure, left ventricle (dP/dt max) / P and left ventricular end-diastolic pressure, which reflect cardiac function, were measured. Left ventricular systolic pressure in sham-operated rats was 139.2 ± 5.2 mmHg (Fig. 4A). Left ventricular systolic pressure in rats with ischemia–reperfusion decreased to 103.4 ± 7.5 mmHg 24 h after

Table 3
Mean blood pressure and heart rate.

	Sham	I/R	+ 10 mg/kg SPZ	+ 30 mg/kg SPZ	+ 10 mg/kg Cime	+ 30 mg/kg Cime
Mean blood pressure (mmHg)	114 ± 2	100 ± 4	115 ± 5	111 ± 2	100 ± 4	111 ± 7
Heart rate (bpm)	437 ± 11	411 ± 10	415 ± 6	419 ± 10	415 ± 5	418 ± 6

Mean blood pressure and heart rate were measured 24 h after reperfusion. Values are mean ± S.E.M. of five separate experiments. Sham: sham-operated group, I/R: ischemia and reperfusion group, SPZ: sulfaphenazole, Cime: cimetidine.

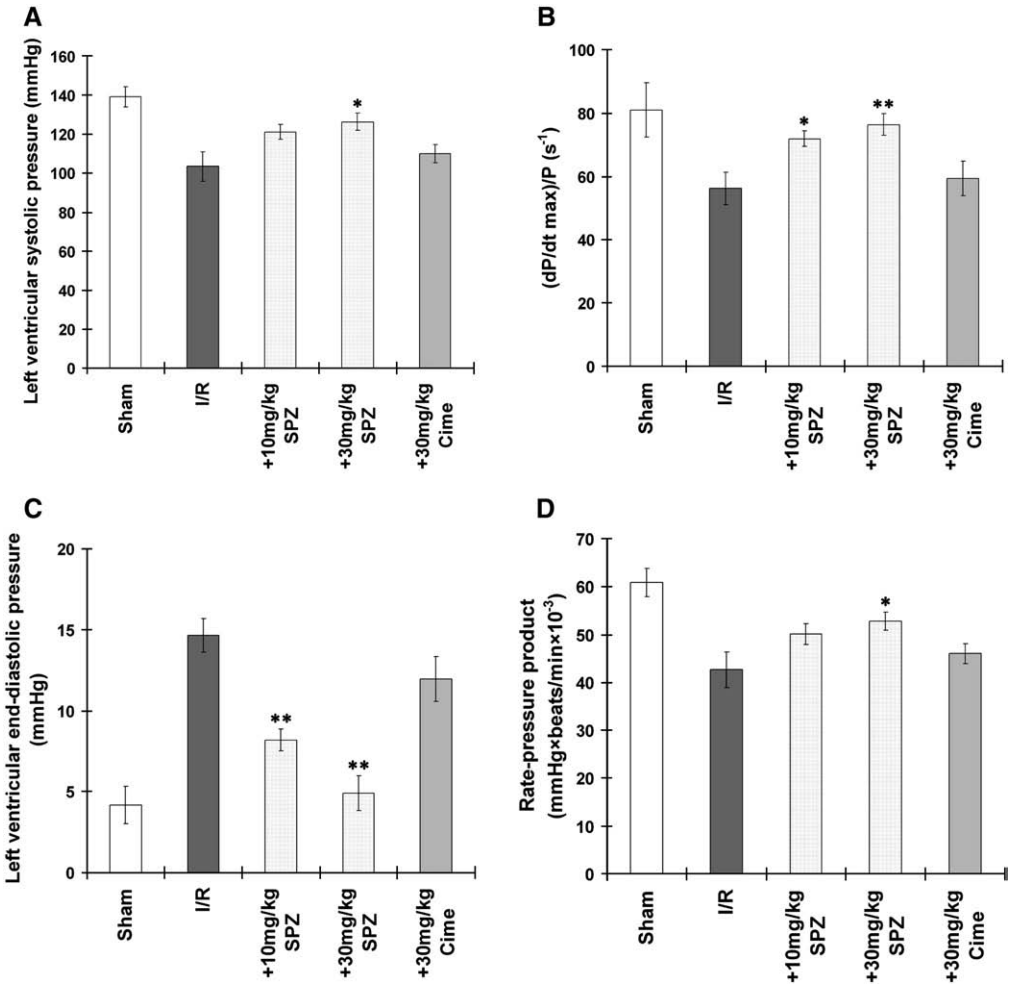

Fig. 4. Improvement of cardiac function by administration of cytochrome *P450* inhibitors. Left anterior descending coronary artery was occluded for 1 h and reperfused for 24 h. (A) Left ventricular systolic pressure, (B) left ventricle (d*P*/d*t* max) / *P* and (C) left ventricular end-diastolic pressure were measured using Millar's catheter inserted into the right carotid artery and advanced to the left ventricle. (D) Rate-pressure product was calculated from the values of heart rate and left ventricular systolic pressure. Values are mean ± S.E.M. of five separate experiments. Data were analyzed using Dunnett's test. **P*<0.05 and ***P*<0.01 vs. I/R group. Sham: sham-operated group. I/R: ischemia and reperfusion group. HR: heart rate, LVSP: left ventricular systolic pressure, SPZ: sulfaphenazole, Cime: cimetidine.

reperfusion. Sulfaphenazole administration improved the decrease in left ventricular systolic pressure in a dose-dependent manner. Left ventricular systolic pressure of rats treated with 30 mg/kg sulfaphenazole was 126.2 ± 4.5 mmHg, which was significantly higher than that of untreated rats. Following reperfusion, left ventricle (d*P*/d*t* max) / *P* decreased gradually with time. The left ventricle (d*P*/d*t* max) / *P* in the ischemia–reperfusion group was about half that of sham-operated rats 24 h after reperfusion (Fig. 4B). Sulfaphenazole administration dose-dependently recovered left ventricle (d*P*/d*t* max) / *P* from 56.2 ± 5.1 to 71.8 ± 2.4 s^{-1} at 10 mg/kg and to 76.3 ± 3.3 s^{-1} at 30 mg/kg, respectively. Left ventricular end-diastolic pressure of rats with ischemia–reperfusion increased to 14.7 ± 2.3 mmHg from 4.2 ± 1.2 mmHg in sham-operated rats (Fig. 4C). Sulfaphenazole administration dose-dependently suppressed the increases in left ventricular end-diastolic pressure to 8.2 ± 0.7 mmHg at 10 mg/kg and 4.9 ± 1.1 mmHg at 30 mg/kg, respectively.

Rate-pressure product, which is an index of myocardial oxygen consumption, in sham group was $6.1 \times 10^4 \pm 0.3 \times 10^4$ mmHg bpm. Rate-pressure product decreased in the ischemic-reperfusion group by 30%. Treatment with sulfaphenazole increased rate-pressure product from $4.3 \times 10^4 \pm 0.4 \times 10^4$ mmHg bpm to $5.0 \times 10^4 \pm 0.3 \times 10^4$ mmHg bpm at 10 mg/kg, and to $5.3 \times 10^4 \pm 0.2 \times 10^4$ mmHg bpm at 30 mg/kg in a dose-dependent manner (Fig. 4D). Another cytochrome *P450* inhibitor, cimetidine, tended to improve the decreases in mean blood pressure, left ventricular systolic pressure, left ventricle d*P*/d*t* max, rate-pressure product and increases in left ventricular end-diastolic pressure.

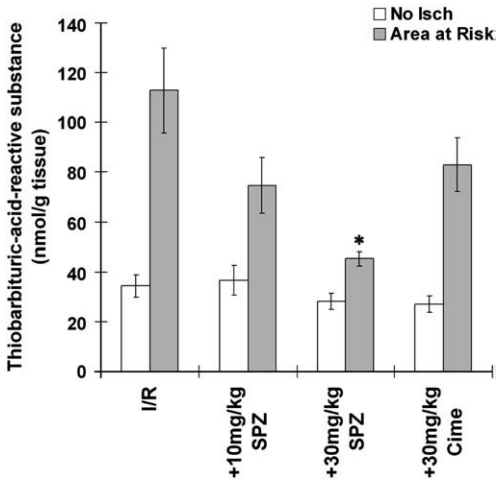

Fig. 5. Suppression of lipid peroxidation by administration of cytochrome *P450* inhibitors. Left anterior descending coronary artery was occluded for 1 h and reperfused for 24 h. The heart was excised, followed by visualization of the area at risk by injection of Evans blue dye. Non-ischemic region (No Isch) and the area at risk were frozen and homogenized, respectively, and the levels of thiobarbituric-acid-reactive substance determined. Values are mean ± S.E.M. of five separate experiments. Data were analyzed using Steel test. **P*<0.05 vs. area at risk of I/R group. Sham: sham-operated group. I/R: ischemia and reperfusion group. TBARS: thiobarbituric-acid-reactive substance, SPZ: sulfaphenazole. Cime: cimetidine.

3.4. Inhibitory effects of cytochrome P450 inhibitors on lipid peroxidation

The heart was excised 24 h after reperfusion, and thiobarbituric-acid-reactive substance contents in the non-ischemic region and the area at risk measured. Thiobarbituric-acid-reactive substance contents in non-ischemic region were about 31.6 ± 4.2 nmol/g tissue in all groups and showed no difference between groups (Fig. 5). In rats with ischemia and reperfusion, thiobarbituric-acid-reactive substance contents in the area at risk were about three times higher than those in the non-ischemic region. Sulfaphenazole administration suppressed the increments of thiobarbituric-acid-reactive substance in a dose-dependent manner, and 30 mg/kg sulfaphenazole significantly reduced thiobarbituric-acid-reactive substance contents from 112.9 ± 17.1 to 45.3 ± 2.9 nmol/g tissue in the area at risk. Administration of cimetidine was also effective in reducing thiobarbituric-acid-reactive substance contents in the area at risk (Fig. 5).

3.5. Decreases in reactive oxygen species levels by administration of cytochrome P450 inhibitors

Next, we examined cardiac reactive oxygen species levels using fluorescent dye dihydroethidium. Fluorescence derived from ethidium, which was formed by the reaction of superoxide anion and dihydroethidium, in non-ischemic region was scarcely detected in all samples. After 10 min reperfusion, marked fluorescence was detected in the nuclei of cardiomyocyte in the area at risk (Fig. 6), indicating the production of reactive oxygen species during reperfusion process. Administration of cytochrome *P450* inhibitor, sulfaphenazole (30 mg/kg), remarkably decreased fluorescence from nuclei. Administration of the same dose of cimetidine (30 mg/kg) was less effective than that of sulfaphenazole in suppression of fluorescence from nuclei.

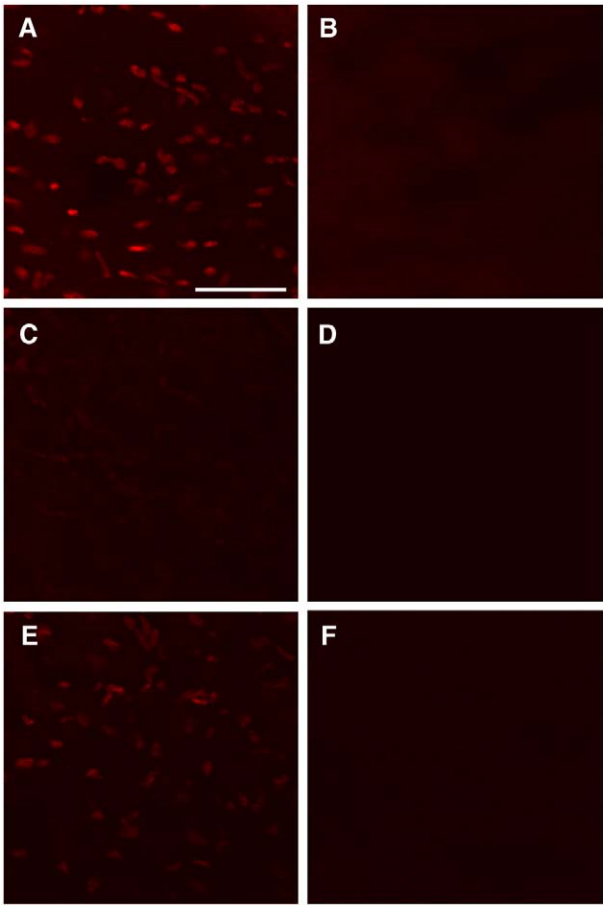

Fig. 6. Decreases in cardiac reactive oxygen species levels by administration of cytochrome *P450* inhibitors. Left anterior descending coronary artery was occluded for 1 h and reperfused for 10 min. Dihydroethidium solution was administrated into left ventricle, and then the heart was isolated, followed by visualization of the area at risk by retrograde injection of Evans blue dye from aorta. The potions of area at risk and non-ischemic region were excised and frozen. Each frozen section was observed under confocal fluorescence microscope. Bars in the micrographs represent 50 μm. (A) The area at risk and (B) non-ischemic region from untreated rats. (C) The area at risk and (D) non-ischemic region from rats treated with 30 mg/kg of sulfaphenazole. (E) The area at risk and (F) non-ischemic region from rats treated with 30 mg/kg of cimetidine.

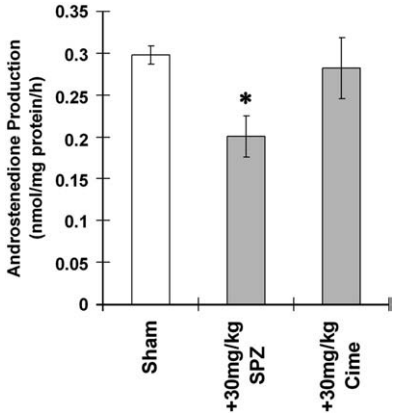

Fig. 7. Suppression of cardiac cytochrome *P450* activities by administration of cytochrome *P450* inhibitors. Following the administration of cytochrome *P450* inhibitors into femoral vein, the heart was excised and microsomal fraction was prepared. Androstenedione production by testosterone metabolism in cardiac microsomes was measured by HPLC method with UV detection. Values are mean ± S.E.M. of four separate experiments. Data were analyzed using the Steel test. *$P<0.05$ vs. Sham group. Sham: sham-operated group, SPZ: sulfaphenazole, Cime: cimetidine.

3.6. Inhibitory effects of sulfaphenazole and cimetidine on cardiac cytochrome P450

Cytochrome *P450* in adult rat cardiomyocytes was reported to metabolize testosterone to androstenedione (Thum and Borlak, 2000). Thus, we measured an androstenedione production in cardiac microsomes. Cardiac microsomes were prepared from the left ventricle 30 min after administration of CYP inhibitors, 30 mg/kg of sulfaphenazole and 30 mg/kg of cimetidine, from the femoral vein. Androstenedione production was 0.30 ± 0.01 nmol/mg protein/h in cardiac microsomes in sham-operated group (Fig. 7). Administration of sulfaphenazole inhibited androstenedione production to 0.20 ± 0.02 nmol/mg protein/h. However, Administration of cimetidine showed almost no effect on that production (0.28 ± 0.04 nmol/mg protein/h).

4. Discussion

Cardiac ischemia–reperfusion injury was induced by the occlusion of left anterior descending coronary artery for 1 h followed by reperfusion for 24 h. After 24-h reperfusion, development of infarct size, cardiovascular dysfunction (as shown by decreases in left ventricular systolic pressure, left ventricle (dP/dt max)/P, rate-pressure product and increases in left ventricular end-diastolic pressure) and increases in lipid peroxidation and levels of reactive oxygen species in the area at risk were observed in untreated rats. Sulfaphenazole administration at the time of reperfusion dose-dependently reduced infarct size and improved cardiac dysfunction. Levels of thiobarbituric-acid-reactive substances and reactive oxygen species in the area at risk decreased after treatment with sulfaphenazole. Administration of cimetidine had a similar, but slightly weaker, effect than sulfaphenazole.

Administration of cytochrome *P450* inhibitors suppressed extension of infarct size and improved cardiac function *in vivo*. Improvement of cardiac function is considered to be due to reduction in infarct

size. This was supported by the data that plasma cardiac troponin I, which is intimately associated with the infarct size, abruptly increased 3 h after reperfusion and increased gradually until 24 h.

Reactive oxygen species are one of the factors responsible for myocardial damage in ischemic and reperfused myocardium. The findings that both thiobarbituric-acid-reactive substance content and ethidium fluorescence in the ischemic region were increased clearly indicate the induction of oxidative injury by reactive oxygen species produced in the ischemic region. This is in line with many reports which show that massive amounts of reactive oxygen species are generated in the ischemic region during reperfusion (Becker, 2004).

Myocardial injury during ischemia–reperfusion has been considered to be due to reactive oxygen species derived mainly from neutrophils that invade the ischemic region (Vinten-Johansen, 2004). Neutrophils are localized to the intravascular space at the early stage of reperfusion, while they are found in the interstitial compartment at later time points (Albertine et al., 1994; Jordan et al., 1999; Rochitte et al., 1998). However, neutrophils detected in the ischemic region 4 h after reperfusion were reported to be as much as one-third of those 1 h after reperfusion (Dreyer et al., 1991), suggesting that reactive oxygen species produced by neutrophils considerably decreased after 4 h of reperfusion. In this study, intravenous administration of sulfaphenazole and cimetidine upon reperfusion was effective in suppressing reperfusion injury, including extension of infarct size, cardiovascular dysfunction, as well as increments in cardiac troponin I contents in plasma and reduced levels of thiobarbituric-acid-reactive substance and reactive oxygen species in the ischemic region. Taken together, these observations strongly indicate that reactive oxygen species produced by cardiac cytochrome *P450*s play a significant part in myocardial injury throughout 24 h of reperfusion. Whether sulfaphenazole or cimetidine inhibits neutrophils should be examined in further studies.

Coronary blood flow after reperfusion is reported to be increased compared with that before ischemia (Zhao et al., 2003). Increment of coronary blood flow leads the increases in oxygen supply to cardiomyocytes. The contents of reactive oxygen species derived from cytochrome *P450*s increased in an oxygen concentration-dependent manner (Ishihara et al., unpublished data; Puntarulo and Cederbaum, 1988) because cytochrome *P450*s always generate reactive oxygen species, including superoxide anion and hydrogen peroxide through an uncoupling reaction (Loida and Sligar, 1993). It is conceivable that the increases in reactive oxygen species produced by cytochrome *P450*s upon reperfusion were due to increases in their uncoupling rate concomitant with the increment of oxygen supply to cardiac tissue.

Sulfaphenazole and cimetidine suppressed reperfusion injury. Both agents inhibit cytochrome *P450* activities, but the suppressive effects of sulfaphenazole on reperfusion injury were more effective than those of cimetidine. Possible reason is that inhibitory effects of sulfaphenazole on cardiac cytochrome *P450* activities are more potent than those of cimetidine. However, to our knowledge, inhibition of the activity of cardiac cytochrome *P450* by sulfaphenazole or cimetidine *in vivo* has not been reported to date. Thus, we examined the inhibitory effects of sulfaphenazole and cimetidine on cardiac cytochrome *P450* activity using cardiac microsomal fraction, which was prepared after intravenous injection of cytochrome *P450* inhibitors. Activity of cardiac cytochrome *P450* was significantly decreased by administration of sulfaphenazole, but administration of cimetidine showed little effect. Therefore, suppressive effects on reduction in infarct size were attributed to the abilities of cardiac cytochrome *P450* inhibition, which was inversely well related to reactive oxygen species levels in ischemic region. This was supported by the findings that sulfaphenazole has high abilities to inhibit cytochrome *P450* activity and to suppress reactive oxygen levels, while both effects of cimetidine are very weak. These facts strongly indicate that reactive oxygen species derived from cytochrome *P450* is responsible for cardiomyocyte injury during reperfusion.

There are a number of reports that reactive oxygen species scavengers suppressed cardiac reperfusion injury. We also reported the inhibitory effects of reactive oxygen species scavengers on reperfusion arrhythmia (Kato et al., 1988; Tada et al., 1990), but most of the reactive oxygen species scavengers were administrated before or during ischemia including our previous studies. In this study, considerable effects on reducing infarct size by post-ischemic administration of sulfaphenazole were recognized, suggesting that the possibility of cytochrome *P450* inhibitors as therapeutic agents for cardiac reperfusion injury.

In conclusion, administration of the cytochrome *P450* inhibitors, sulfaphenazole and cimetidine, upon reperfusion, suppressed the development of infarct size and improved the declined cardiac function in a regional ischemia–reperfusion model. The *in-vivo* ischemia–reperfusion model used in this study was considered to be closer to clinical settings. These results suggest that cytochrome *P450* inhibitors may be therapeutic agents for cardiac reperfusion injury.

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